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A SIMPLE SINGLE-CELL TECHNIQUE FOR GENETIC STUDIES OF BACTERIA

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Numerous techniques have been described for the propagation of bacterial cultures from individual cells. (See Hildebrand, 1950, for a review.) Although isolation of single cells may result in genetic homogeneity in the few generations immediately following their isolation, spontaneous mutations almost certainly destroy this homogeneity upon continued cultivation. Consequently, the description of another technique for obtaining such single-cell cultures would serve little purpose. The present paper describes an extension of the usual techniques that makes possible critical genetic studies when applied to suitable material. This extension consists of the isolation of a number of single-cell cultures of known relationships descending from one original cell.

SINGLE-CELL ISOLATION TECHNIQUE

A film of agar is prepared by spreading about 0.05 ml of melted 2 per cent agar with a heated, 0.1-ml graduated pipette over the central portion of a 22-by-50-mm no. 1 cover slip. The cover slip is inverted over a moist chamber of about 10-mm depth, which is closed at one end and contains strips of wet blotting paper to prevent drying. Any medium supporting growth of the particular organism may be employed and may be satisfactorily clarified by centrifugation.

A regular pattern of reference holes is then punched in the agar film with a sterile micropipette of 10 to 30 microns in outside diameter. The pattern used by the writer is shown in figure 1 along with the numbers of the progeny cells that will ultimately be placed at the positions shown. Up to 12 such patterns, each providing for 16 cells, may be accommodated upon the agar film. The holes are spaced about $\frac{2}{3}$ the width of the field of a 44× objective apart, thus permitting easy location of cells at given positions.

The method employed by the writer to record relationships among the progeny cells is indicated in figure 1. The initial cell, 0, divides into cells 1 and 2. Cell 1 divides into cells 3 and 4, etc. The progeny of any cell, n, are $2n + 1$ and $2n + 2$. Zelle and Lederberg (1951) present clonal pedigrees obtained by this technique. When only one initial cell is used for the entire film, as many as 150 related single-cell cultures may be obtained from one initial cell. Except for the first few divisions, only one member of each pair of sib cells must be moved to a new position. Since the location of each cell is predetermined, little time is lost in moving cells to their new positions or in relocating cells once they have been placed in their positions. The time at which each cell is separated and placed in its position is recorded.

With another sterile micropipette having a lumen of 10 to 15 microns in di-

ameter, a few cells are deposited near the center of the pattern of reference holes. This is accomplished by allowing the pipette to fill by capillarity from a liquid suspension or culture of bacteria and then touching it lightly to the surface of the agar film. Since any excess moisture in the microdrop is quickly absorbed by the agar, the cells all lie in a horizontal plane with no Brownian movement.

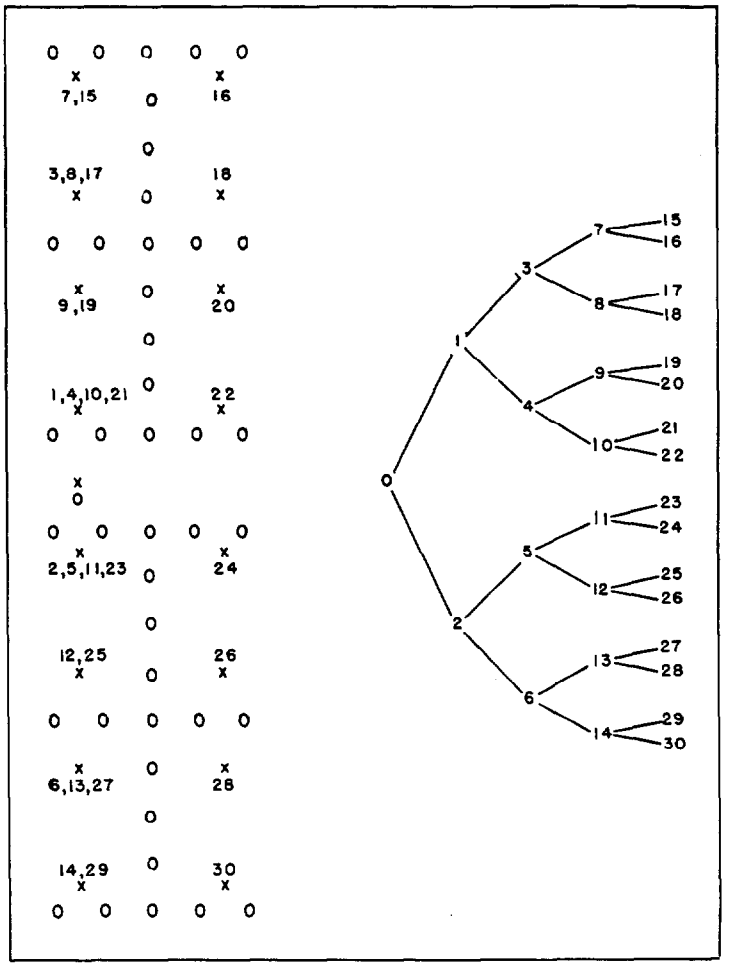

Figure 1. Pattern for reference holes (O) with the number of the cells that will be placed at the predetermined locations (X). Pedigree on right shows the relationships of the cells.

These factors permit more critical observation. The lag period before the first division occurs may be reduced if the cells deposited are from a culture in the logarithmic phase of growth.

Following deposition of the microdrop, a number of individual cells are teased into various central positions by means of a sterile microneedle about 3 to 5 microns in diameter with a smooth tip. This same microneedle is used in separating the daughter cells following division and moving them to their predetermined

positions. The cells may be moved quite easily by appropriate movements of the microscope stage after touching the needle lightly to the agar near by. After several individual cells have been put in position, the moist chamber is incubated in a petri dish at the appropriate temperature. After the first division of the individually positioned cells, one or more may be selected as the initial cell

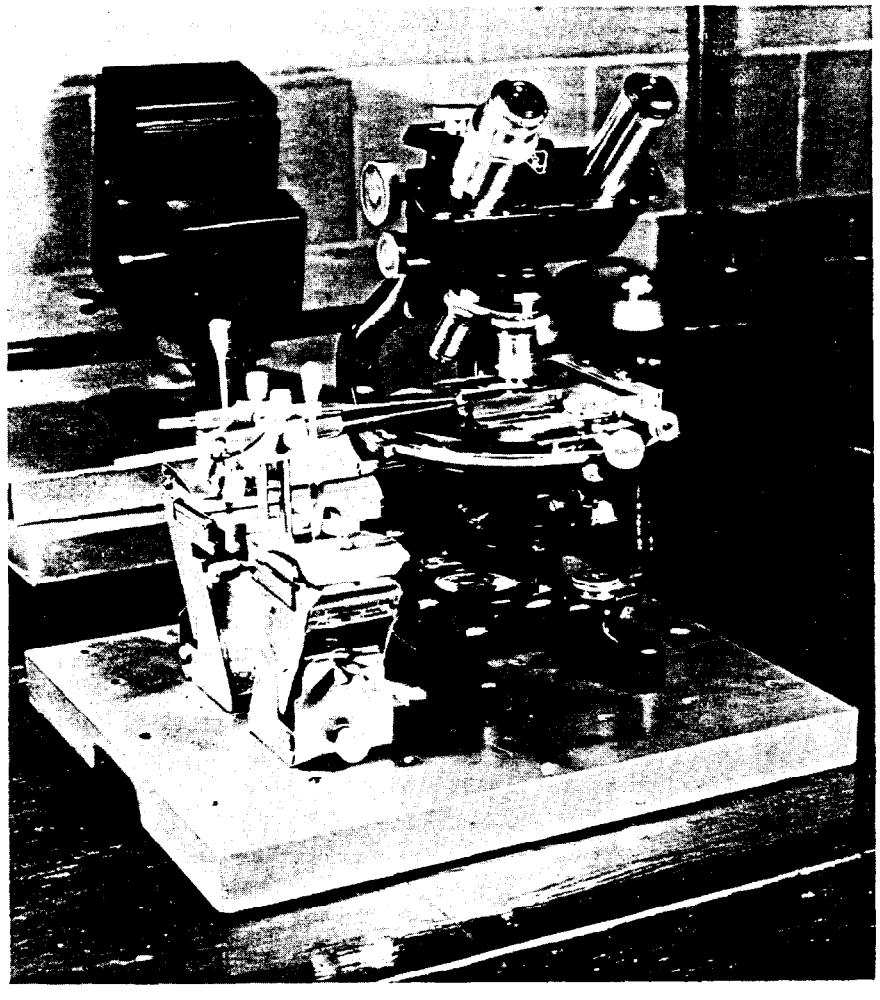

Figure 2. Photograph of microscope and micromanipulator arrangement.

or cells and the two daughter cells of each moved to predetermined locations in the pattern. The excess cells are then picked up with another sterile micropipette of about 10 microns in inside diameter. This is done by touching the pipette to the agar near the cells. Capillarity will cause the cells to flow into the pipette. Thus the only cells remaining upon the agar film are those whose subsequent progeny will continue to be separated in the manner indicated above.

Finally, after the desired number of progeny cells have been derived from

each initial cell, the moist chamber is incubated and each cell allowed to develop into a microcolony. Each microcolony is then transferred to 0.5 ml of liquid medium and incubated further. Large micropipettes that permit their contents to be expelled by blowing with the lips are used to transfer the microcolonies. If these transferring pipettes are rinsed in alcohol soon after use to prevent plugging, they may be autoclaved and used repeatedly, thus removing most of the tedious drawing of micropipettes. The microcolonies can be successfully transferred when as few as 16 cells are present, but allowing them to develop further ensures successful transfer. Since every cell in the microcolony can be picked up, quantitative studies may be made if desired.

Since most of the actual moving of the cells is done with the microscope stage, any binocular microscope with a good, preferably rotating, mechanical stage may be used. The writer has found the Bausch and Lomb, model DDE, most convenient. An Abbe 1.40 NA condenser with the top lens removed and 15× eyepieces are employed. Although a 1.25 NA oil immersion objective can be employed if necessary, the greater working distance of an achromatic, 4-mm 0.65 NA objective is convenient. A parfocal, 16-mm objective is used to center micropipettes in the field and for most operations other than the actual separation of the sister cells. Figure 2 shows the micromanipulator and microscope arrangement.

Very elaborate micromanipulators are not required since only the vertical movement is critical. The Chambers and Emerson designs have been very adequate. A single unit is sufficient, although it is sometimes convenient to use a small microneedle for separating the sib cells and a larger one for moving them to their new positions.

The repeated use of the microneedles for separation and the micropipettes for transferring microcolonies to liquid media reduces to a minimum the time spent in making micropipettes. The writer makes pipettes by first drawing 4- or 5-mm pyrex or soft glass tubing to a capillary and then drawing the capillary portion to a fine point in the flame of a microburner having a blunt 26-gauge hypodermic needle for a tip. Solid glass rod is used for making the microneedles. The large pipettes used for transferring the microcolonies are most easily made by drawing the capillary to a fine tip and then breaking it after nicking it with the edge of a fragment of a carborundum glass-cutting wheel.

DISCUSSION

The method described is useful not only in making large numbers of single-cell cultures but also in making genetic studies of bacterial strains that mutate or segregate at high rates. Zelle (1942) showed that the change from a smooth to a rough colony type in an unstable strain of *Salmonella typhimurium* could best be interpreted as a very high mutation rate and could not be due to somatic crossing over. Zelle and Lederberg (1951) have employed the method in studies of segregation in semipermanent heterozygous cells formed by sexual fusion in the K-12 strain of *Escherichia coli*.

The method is being further extended in order to permit a critical cytological

study of bacteria. Individual cells in known stages of the division cycle can be transferred to a cover slip. If some means can be found to prevent the too high frequency of loss of cells during the staining procedures, it should be possible to study accurately the method of division and distribution of nuclear bodies to the daughter cells.

SUMMARY

A simple technique for isolating large numbers of single-cell cultures with known relationships is described, and applications of the technique in genetic studies of bacteria are discussed.

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